Submitted by Vejay K. Ravindran in partial fulfillment of the requirements for the degree of Master of Science specializing in Oral Biology.

Accepted on behalf of the Faculty of the Graduate School by the thesis committee:

10 June 16
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To compare the effects of storage solutions 0.05% Thymol, vs. 6% Sodium Hypochlorite vs. Hank's Balanced Salt Solution on the flexural strength of dentin bars.

BY

Vejay K. Ravindran

Submitted in partial fulfillment of the requirements
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Introduction

Statement of the problem:

Prior to use in vivo, dental materials are routinely tested in vitro utilizing human extracted teeth. After extraction, teeth must be disinfected and stored until use. However, the solutions used to disinfect and store teeth after extraction may alter their internal structure. Any changes in the internal structure of an extracted tooth, used to test dental materials in vitro could result in findings that cannot be replicated or generalized to teeth in vivo. If extracted teeth are to be treated as valid proxies of teeth in vivo, it is critical to understand the effect of disinfection and storage solutions on the internal tooth structure of extracted teeth.

In vitro tests are valuable to predict characteristics of newer materials. Dentin, the inner layer of human teeth has been the subject of many tests evaluating its properties including dentin permeability (Goodis HE, 1993) and flexural bond strength (Grigoratos, 2001). Dentin constitutes the largest portion of the tooth and consists of approximately 70% inorganic matter and 30% organic matter and water. Collagen is the major protein found in dentin. It constitutes 90% of the organic matrix. Dentin continues to develop throughout life (Ten Cate, 1994). Stimuli such as dental caries or attrition of teeth can cause new formation of dentin. It is important to recognize that the composition of dentin is not static. It is influenced by the relative position of the dentin within the tooth, the age of the dentin, and the presence and/or absence of disease. Toto found that teeth over 50 years old contained less water than young teeth (10-20 years of age) (Toto, 1971). Dehydrated dentin has lower toughness and has been described as being brittle (Jameson MW, 1993).

Researchers often test various characteristics of material strength and use dentin to simulate in vivo conditions. Flexural strength or the fracture resistance of dentin is a common testing parameter, and is measured under laboratory conditions via the three point loading test. This test can determine the flexural properties of dentin in the

form of rectangular bars. The basis of using rectangular bars was developed using the American Society of Testing and Materials as a guide. (American Society for Testing and Materials, 1989). The three point bend test provides values for flexural stress, flexural strain, and modulus of rupture. It is commonly performed by a universal testing machine (*5940 Single Column Tabletop Testing System, Instron,* Norwood, MA, USA). D. Arola tested the flexural strength of dentin using the three point loading test and concluded the dentin becomes more brittle with age (Arola, 2004). Reported values for flexural strength for dentin range from 245 to 280 Mpa (Waters, 1980). Flexural Strength is the gold standard parameter to observe alterations of mechanical properties in mineralized tissues and a good predictor of the other important mechanical properties such as Young's modulus and yield stress. (Aydin B, 2015)

For in vitro measures such as flexural strength to be considered valid proxies of in vivo conditions, materials must be tested on teeth that closely approximate those found in the human mouth. Extracted teeth not used immediately must be cleaned, disinfected, and stored until used in research. Research has shown that using the disinfectant solution as the storage medium is the most common protocol. The Center for Disease Controls (CDC) suggests that before extracted teeth are manipulated the teeth first should be cleaned by scrubbing with disinfectant and water or by using an ultrasonic cleaner. After disinfection and cleaning, teeth should be stored in a liquid chemical germicide solution suitable for clinical specimen fixation. The CDC cautions against autoclaving teeth when used for laboratory testing because of the potential to alter tissue properties (Centers for Disease Control and Prevention. Recommended infection-control practices for dentistry, 1993). The ideal germicide would disinfect and hydrate extracted teeth during storage without further altering tissue structure. Researchers have questioned whether the germicides used as storage mediums could alter the properties of dentin and other tooth tissues, and to date there is no standard protocol for disinfecting and storing extracted human teeth. Disinfectant solutions used as storage solutions in previous studies include saline (Tao L, 1998), sodium Hypochlorite (Grigoratos, 2001), thymol (B.H Kivanc, 2009),

and Hanks Balanced Salt Solution (Aydin, 2015), but the ability of these storage media to alter the flexural strength of dentin has received little investigation. Grigoratos tested the effect of 3% and 5% sodium hyochlorite on dentin bars. He found that immersion of sodium hypochlorite on dentin decreased the flexural strength of dentin. He postulated that sodium hypochlorite might interfere with the collagen composition of dentin making it brittle (Grigoratos, 2001). Moura found that sodium hypochlorite may alter dentin structure by removing or modifying the proteic matrix. (Moura, 2004) S. V Barbosa determined that 5% Sodium hypochlorite is capable of extracting organic material from dentine tissue (Barbosa, 1994). Therefore, previous studies have demonstrated that disinfectant and storage media can alter the internal properties and flexural strength of dentin.

A commonly used storage and disinfection media is Thymol; a naturally occurring biocide with strong antimicrobial attributes (Stappert, 2006). Shafiei used 0.5% thymol solution as a storage medium before testing the flexural strength of extracted premolars.(Shafiei, 2014). To date research has not tested to determine if Thymol affects the dentin to reduce the flexural strength.

One of the drawbacks of all storage solutions is the failure to simulate in vivo conditions. Hanks Balanced Salt solution (HBSS) provides cells with water and inorganic ions, while maintaining a physiological pH and osmotic pressure. HBSS contains sodium, potassium, calcium, and chloride. The solution is commonly used to preserve periodontal ligament cells on a tooth that has been avulsed from the patient's mouth (Aydin, 2015). The ability of HBSS to prevent periodontal ligament cell death demonstrates that it provides a reasonable facsimile of intraoral conditions.

The way in which extracted teeth used as a dentin substrate are cleaned, disinfected, and stored prior to use in research may alter the dentin and thus adversely impact the generalizability of in vivo findings to in vitro applications. Therefore, it is critical to understand the effect of solutions used to disinfect and store extracted teeth on dentin

structure. A gap in the literature is that the effect of storage solutions including thymol, and sodium hypochlorite on the flexural strength of dentin is unknown.

This study will address that gap by comparing the flexural strength of dentin stored in 0.05% Thymol *(Thymocide, Wexford Labs, Kirkwood, MO, USA)*, versus dentin stored in 6% sodium hypochlorite (*6% Sodium Hypochlorite, Vista Dental Products, Racine, WI)*.

Significance:

The ideal storage medium should disinfect and hydrate teeth, without altering the tissue properties of dentin. This study looks at the potential of storage mediums to alter the physical properties of dentin. The results could help researchers who study the flexural strength of the dentin to select an appropriate storage medium.

Purpose

To determine if dentin bars stored in storage solutions 0.05% Thymol, and 6% Sodium Hypochlorite could alter the flexural strength of dentin bars when compared to dentin bars immersed in Hanks Balanced Salt Solution.

To compare the effects of 0.05% Thymol versus 6% sodium hypochlorite versus Hands Balanced Salt Solution used as a storage solution on the flexural strength of dentin.

Hypothesis

1. Dentin bars treated with 0.05% thymol will reduce flexural strength of dentin compared to dentin bars treated with Hank's Balanced Salt Solution.

2. Dentin bars treated with 6% sodium hypochlorite will reduce flexural strength compared to dentin bars treated with Hank's Balanced Salt Solution.

Study Design:

Thirty extracted human teeth which included maxillary and mandibular third molars, (n=30) without caries or restorations were extracted from patients 18-36 years of age. Immediatley after extraction blood, and gross debris was wiped off with Cavicide Wipes. (Cavicide Wipes, Meterex Company, Orange, CA USA,). Teeth were placed in Hanks Balanced Salt Solution (*GIBCO® HBSS Grand Island, NY, USA).* This was done until enough teeth for the sample size was collected.

Dentin bars measuring 1mm x 1mm x 10m were cut using a diamond saw (*EC 330 Mini Saw New Exakt Jacksonville, FL, USA*). The bars were measured with a digital caliper (*General 14712 12" Fraction and Digital Fractional Caliper, Chicago, IL, USA*) and randomly assigned to one of four groups. Twenty samples were placed in a solution of 0.05% thymol (Group 4), twenty samples were placed in 6% Sodium hypochlorite (Group 3), and twenty control samples placed in Hank's Balanced Salt Solution (Group 2). Group one contained twenty samples that were immersed in 3% Glutaraldehyde *(MetriCide Co. Orange, CA, USA*). The sample was included for a follow on research project. Data from this sample is included but is not compared in the results of this paper. All samples were immersed for two hours as followed by the Grigoratos study. (Grigoratos, 2001)

Dentin bars were subjected to three point bend tests, using a test jig mounted on a load testing machine -Instron Machine. The Instron Machine (*5940 Single Column Tabletop Testing System, Norwood, MA)* was calibrated before testing per manufacturer guidelines. The load testing machine was run at a cross head speed of 0.5mm min to failure. The primary outcome was the mPA reading on the Instron guage at which each sample fails.

Preparation of test Solutions:

The storage solutions were obtained from the following manufacturers:

1. Hanks Balanced Salt Solution (*GIBCO® HBSS Grand Island, NY, USA*)
2. Thymol 0.05% (*Thymocide, Wexford Labs, Kirkwood, MO, USA)*
3. Sodium Hypochlorite *(6% Sodium Hypochlorite, Vista Dental Products, Racine, W)*

Data Analysis and Statistics:

Data was examined for normality and outliers both graphically using histograms and normal quantile plots, and using the Kolmogorov-Smirnov and Shapiro-Wilk tests for normality. Transformations (e.g. natural log) were applied when required. Mean and standard deviation of flexural strength was calculated for each condition, and the 6% NaOCL and Thymol 0.5% conditions was compared with the HBSS condition using Student's t test for independent samples. If data did not meet the assumptions of t tests or ANOVA even after transformation, appropriate nonparametric tests (Mann-Whitney U for 2 groups, Kruskal-Wallis test for 3 or more groups) was used instead. A 5% two-sided significance level was used.

Sample Size:

With the proposed sample size of 20 per group, the study has 80% power to detect a difference of .91 standard deviations between groups based on a t test for independent samples with a 5%, two-sided significance level.

Figure 1: Flow Chart of Study

Test	Medium	No. of Specimens
Group 1	Glutaraldehyde	20
Group 2 Control	Hank's Balanced Salt Solution	20
Group 3	6% NaOCL	20
Group 4	Thymol 0.5%	20

Statistical Analysis:

Methods: Data did not follow a normal distribution, and could not be transformed to achieve normality and constant variance. Therefore, data are summarized as median (interquartile range) and solutions were compared using the non-parametric Kruskal-Wallis test, followed by Dunn's post hoc tests to adjust for multiple comparisons. SPSS version 22 (© IBM Corp.) was used for statistical analysis.

Results: Load, flexure stress, and time at break all varied significantly by solution ($P < .005$, Kruskal-Wallis test). Median load at break and flexure stress at break were significantly higher for solution 1 than for solution 3 ($P < .001$) and solution 2 ($P = 0.020$) but not solution 4 ($P = 0.071$). There was no significant difference in load or flexure stress among solutions 1, 2 and 4. Median time at break was significantly greater for solution 3 than for solution 1 ($P = .004$), but no other significant differences were observed.

(*NOTE: The median and IQR are in the Statistics table below – median is percentile 50 and IQR is percentile 25-percentile 50, e.g. load at break, solution 1: 15.65 (12.95-21.91). P values comparing the solutions come from the "Adj. Sig." column in the tables labeled "Pairwise Comparisons of Solution." Also, load and flexure stress are a linear function of each other so statistically it's redundant to report both of them*.

Statistics

	Solution	Minimum	Maximum	Percentiles 25	50	75
Load at Break (Standard)	1	10.20331	32.91898	12.9521750	15.6509100	21.9090450
	2	-4.75780	37.97853	-.3782775	11.2979950	15.5748350
	3	-.49270	14.66929	-.3212600	-.1748600	5.1762800
	4	-.53640	45.07882	1.7047700	11.5006350	16.5617675
Flexure stress at Break (Standard)	1	15.30497	49.37848	19.4282650	23.4763650	32.8635650
	2	-7.13670	56.96780	-.5674150	16.9469900	23.3622450
	3	-.73905	22.00393	-.4819000	-.2623000	7.7644100
	4	-.80459	67.61823	2.5571575	17.2509600	24.8426550
Time at Break (Standard)	1	.1	1.3	.275	.431	.832
	2	.3	6.6	.353	.951	3.830
	3	.1	4.1	.559	2.829	3.100
	4	.2	5.1	.323	.579	2.969

Nonparametric Tests

Hypothesis Test Summary

	Null Hypothesis	Test
1	The distribution of Load at Break (Standard) is the same across categories of Solution.	Independent-Samples Kruskal-Wallis Test
2	The distribution of Flexure stress at Break (Standard) is the same across categories of Solution.	Independent-Samples Kruskal-Wallis Test
3	The distribution of Time at Break (Standard) is the same across categories of Solution.	Independent-Samples Kruskal-Wallis Test

Hypothesis Test Summary

	Sig.	Decision
1	.000	Reject the null hypothesis.
2	.000	Reject the null hypothesis.
3	.005	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .050.

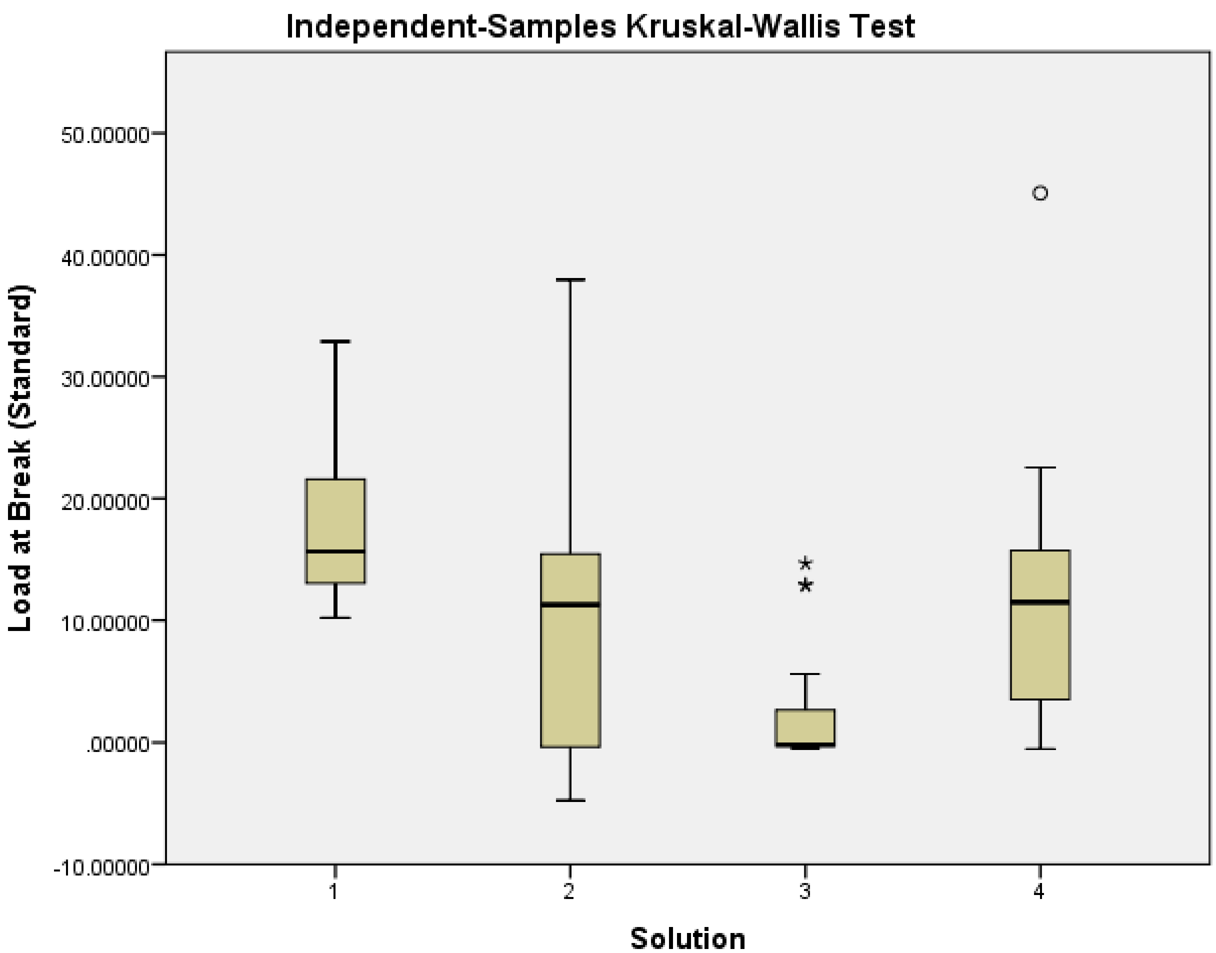
Independent-Samples Kruskal-Wallis Test

Load at Break (Standard) across Solution

Independent-Samples Kruskal-Wallis Test Summary

Total N	79
Test Statistic	23.861[a]
Degree Of Freedom	3
Asymptotic Sig.(2-sided test)	.000

a. The test statistic is adjusted for ties.



Pairwise Comparisons of Solution

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.
3-2	14.316	7.352	1.947	.052	.309
3-4	-18.516	7.352	-2.518	.012	.071
3-1	35.566	7.352	4.838	.000	.000
2-4	-4.200	7.257	-.579	.563	1.000
2-1	21.250	7.257	2.928	.003	.020
4-1	17.050	7.257	2.349	.019	.113

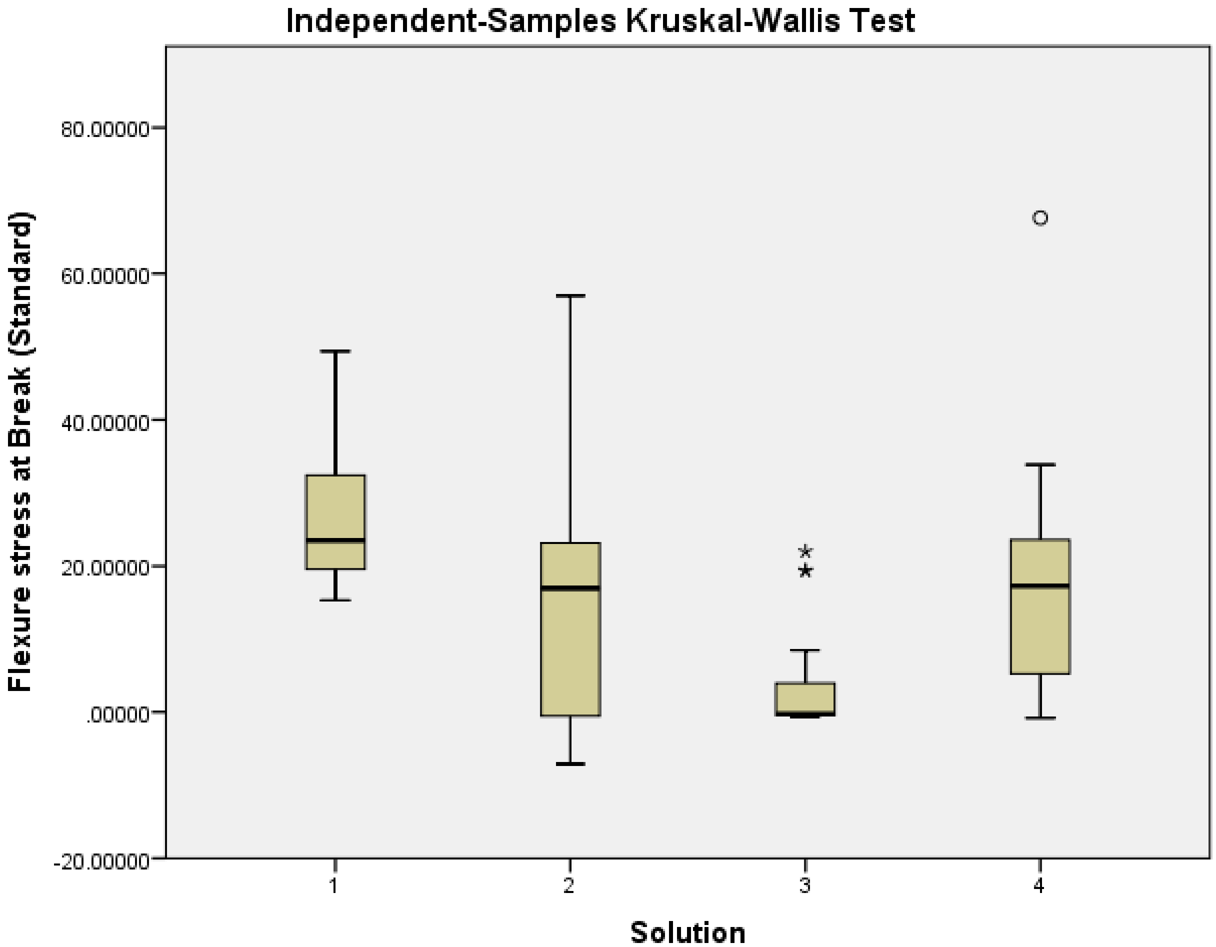
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Flexure stress at Break (Standard) across Solution

Independent-Samples Kruskal-Wallis Test Summary

Total N	79
Test Statistic	23.861[a]
Degree Of Freedom	3
Asymptotic Sig.(2-sided test)	.000

a. The test statistic is adjusted for ties.



Pairwise Comparisons of Solution

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.
3-2	14.316	7.352	1.947	.052	.309
3-4	-18.516	7.352	-2.518	.012	.071
3-1	35.566	7.352	4.838	.000	.000
2-4	-4.200	7.257	-.579	.563	1.000
2-1	21.250	7.257	2.928	.003	.020
4-1	17.050	7.257	2.349	.019	.113

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.

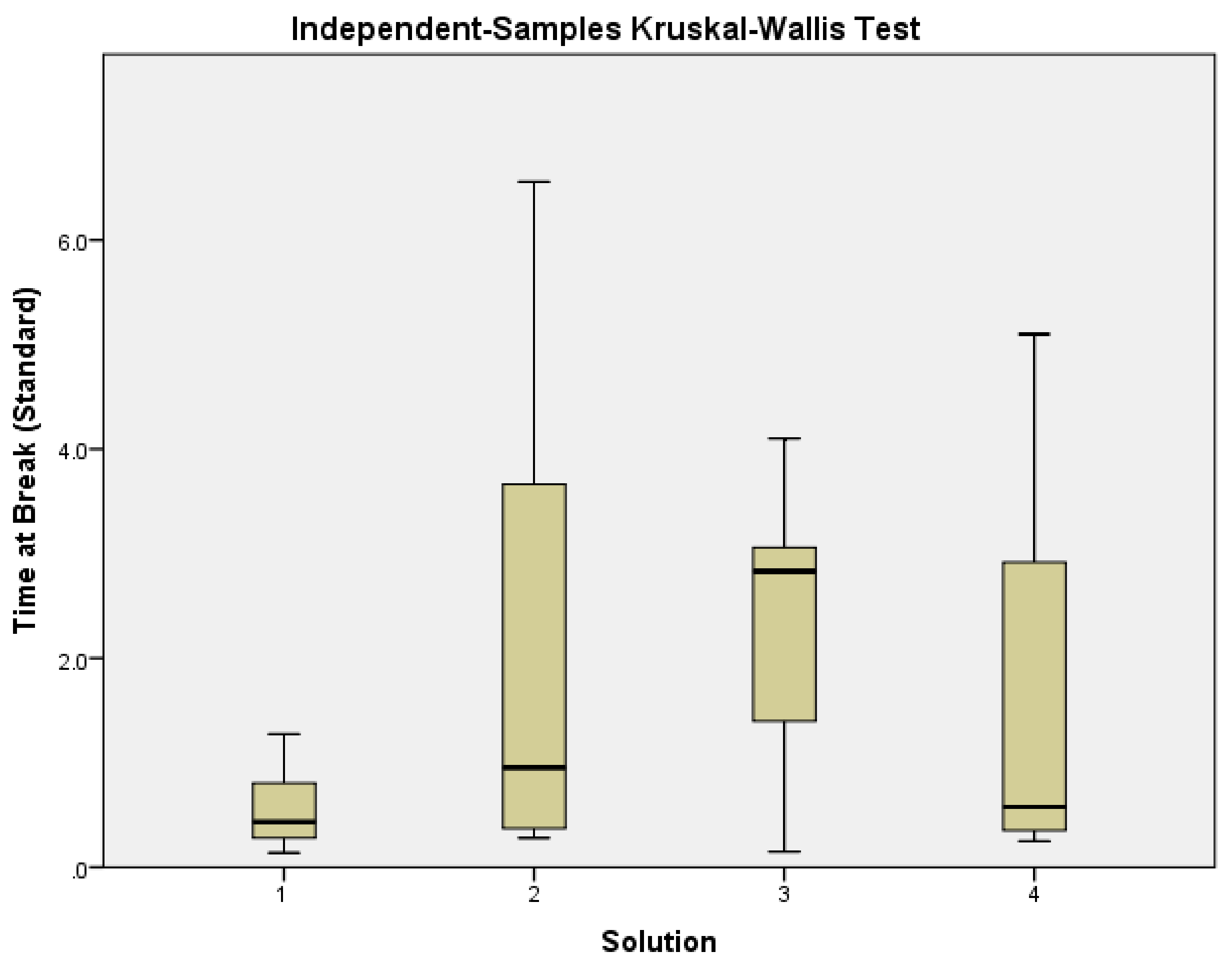
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Time at Break (Standard) across Solution

Independent-Samples Kruskal-Wallis Test Summary

Total N	79
Test Statistic	12.999[a]
Degree Of Freedom	3
Asymptotic Sig.(2-sided test)	.005

a. The test statistic is adjusted for ties.



Pairwise Comparisons of Solution

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.
1-4	-11.125	7.257	-1.533	.125	.752
1-2	-18.825	7.257	-2.594	.009	.057
1-3	-25.021	7.352	-3.403	.001	.004
4-2	7.700	7.257	1.061	.289	1.000
4-3	13.896	7.352	1.890	.059	.352
2-3	-6.196	7.352	-.843	.399	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Discussion

Dentin the inner layer of human teeth has been tested in a variety of research studies. This study attempted to determine if storage solutions used to disinfect and hydrate extracted teeth could alter the properties of dentin. Current research protocols have not identified the ideal storage solution for extracted teeth. Research studies have used a variety of storage solutions including Sodium Hypochlorite, Glutaraldehyde, and Thymol. It's not known if Storage solutions could potentially alter the properties of dentin. In this study hypothesis 1 was rejected. The flexural strength of dentin bars immersed in Thymol was not statistically significantly different than the controls. However hypothesis 2 was correct in that the flexural strength of dentin bars immersed in Sodium Hypochlorite was statistically significantly lower than the control. Previous research has documented that Sodium Hypochlorite can remove the protein matrix from dentin reducing the flexural strength. This was confirmed in this study. Flexural strength of dentin bars immersed in Thymol were similar to HBSS. Sample size included third molars that were fully impacted, partially impacted, and erupted. It's not known if the dentin in third molars is consistent with teeth that are in constant occlusion and subject to reparative dentin.

Within the limitations of this study, teeth stored in Thymol for 2 hours did not show an alteration in the flexural strength of dentin. From the results I believe that

the data presented in this paper have developed useful references for research studies involving extracted teeth.

Conclusion:

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In conclusion this research determined that Sodium Hypochlorite will lower the flexural strength of dentin. Extracted teeth stored in Thymol had flexural strength similar to the control solution. Within the limitations of this study 0.5% Thymol used as storage solution for extracted teeth will not lower the flexural strength of dentin.

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